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(54) Titre : PHOSPHATASES ALCALINES THERMOSTABLES DE RHODOTHERMUS MARINUS

(54) Title: THERMOSTABLE ALKALINE PHOSPHATASE OF RHODOTHERMUS MARINUS

(57) Abrégé/Abstract:

An enzymatically active portion of a thermostable alkaline phosphatase, wherein the polymerase has at least 75% homology in its amino acid sequence to the enzymatically active portion of the alkaline phosphatase of Rhodothermus marinus.



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Abstract

An enzymatically active portion of a thermostable alkaline phosphatase, wherein the polymerase has at least 75% homology in its amino acid sequence to the 5 enzymatically active portion of the alkaline phosphatase of Rhodothermus marinus.

DESCRIPTION

**THERMOSTABLE ALKALINE PHOSPHATASE
OF RHODOTHERMUS MARINUS**

Background of the Invention

Alkaline phosphatases are commonly used in routine biochemical procedures to remove phosphate groups from the
5 termini of nucleic acid molecules. For example, calf intestinal alkaline phosphatase is a heat labile enzyme which is used to remove such phosphate groups, and then is inactivated by exposure to a high temperature. This thermal instability is advantageous because the alkaline
10 phosphatase need not be removed from the reaction mixture prior to subsequent manipulations.

Alkaline phosphatase is also used as a non-radioactive marker for the detection of specific protein or DNA targets. It is conjugated to proteins or
15 DNA oligonucleotides to aid in detection of such targets. Enzyme thermostability is desirable for use in labelling DNA larger than 50bp, and may be useful in other applications for increased stability of the enzyme during storage.

20 Alkaline phosphatases from various thermophilic and other organisms are known: Yeh and Trela (1976) "Purification and Characterization of a Repressible Alkaline Phosphatase from *Thermus aquaticus*" J. Biol. Chem. 251:3134-3139; Hartog and Daniel (1992) "An Alkaline

Phosphatase from *Thermus* sp Strain Rt41A" Int. J. Biochem. 24:1657-1660; Schaffel and Hulett (1978)
"Alkaline Phosphatase from *Bacillus licheniformis*"
Biochimica et Biophysica Acta 526:457-467;
5 Hulett-Cowling and Campbell (1971) "Purification and Properties of an Alkaline Phosphatase of *Bacillus licheniformis*" Biochem. 10: 1364-1371.

Summary of the Invention

10 Applicant has isolated and purified a novel alkaline phosphatase from the thermophilic species Rhodothermus marinus. This enzyme has a high pH optimum (~10.8) and is thermostable, retaining 60% of its activity even after 24 hours incubation at 65°C. This enzyme is also tolerant of
15 other denaturing conditions, including overnight incubation in 6M urea at 65°C. The higher pH optimum of this enzyme is a significant advantage. This high pH optimum, and thus stability at high pH, enhances the use of the enzyme in non-radioactive detection systems, for
20 example, when the enzyme is used with streptavidin. In addition, the high pH optimum of the enzyme makes it suitable for use with dioxetane substrates which undergo rapid conversion to the luminescent form at alkaline pH.

The thermostability of the alkaline phosphatase is
25 also advantageous in that following direct cross-linking of the enzyme to nucleic acid probes, it allows hybridization and subsequent washes of such labelled probes under stringent conditions, that is, at elevated temperatures without significant loss of enzyme activity.

Thus, in a first aspect the invention features an enzymatically active portion of the thermostable alkaline phosphatase present in Rhodothermus marinus having a pH optimum greater than 10.5, e.g. 10.8, which is also
5 resistant to a temperature of at least 65°C (i.e., maintains at least 10% of its activity at this temperature).

By phosphatase is simply meant a protein or fragment thereof having an activity which removes a phosphate group
10 from a molecule, such as a DNA molecule or another molecule, such as p-nitrophenyl phosphate (pNPP). An "alkaline phosphatase" is one which is active at a pH greater than 7, and in the present invention has a pH optimum greater than 10.0 using the conditions listed
15 below. Those skilled in the art recognize that pH optimum is dependant on buffer conditions and substrate concentration.

By "thermostable" is meant that the enzyme maintains at least 10% of its activity after heating at 65°C for one
20 hour or longer, preferably for 5 or 10 hours.

While applicant provides one example of an alkaline phosphatase of the present invention, those in the art armed with the fact that an alkaline phosphatase having a pH optimum of about 10.8 exists in nature and can be
25 isolated can now readily screen cellular extracts to determine the presence of such an activity, and can use standard methodology as described herein to isolate and purify such an enzymatic portion.

The invention also encompasses other enzymatically

active thermostable alkaline phosphatases which have at least 75% homology to the enzymatically active portion of the alkaline phosphatase of Rhodothermus marinus shown in Figure 9 (SEQ. ID. NO. 4). By 75% homology is meant that
5 the thermostable alkaline phosphatase can have an amino acid sequence which differs from that of Rhodothermus marinus by as much as 25 % and still maintain substantially the same enzymatic activity (i.e., the ability to remove a phosphate group from a molecule) as
10 the enzymatically active portion of the alkaline phosphatase of Rhodothermus marinus. Such differences include conservative changes, additions, deletions, and substitutions other than degenerate codons. In preferred embodiments, the amino acid sequence of the alkaline
15 phosphatase includes less than 10 conservative amino acid changes or less than 10 additional amino acids compared to the enzymatically active portion of the alkaline phosphatase of Rhodothermus marinus. By substantially the same enzymatic efficiency is meant at least 50% of the
20 activity of the enzymatically active portion of the thermostable alkaline phosphatase of Rhodothermus marinus.

In the present invention, the enzyme is preferably provided in a purified form, that is, it is isolated from
25 the environment in which it naturally occurs. Generally, such an environment is within a bacterial cell and the protein is isolated from the cell wall and/or membranes of that cell such that it is enriched at least 10- or 100-fold compared to its presence in the cell. More

preferably, it is enriched 1000- or 10,000- or more fold such that it is an essentially homogeneous preparation, that is, it is the predominant species of protein in a preparation. Even more preferably, the protein is the
5 only species, that is, it represents at least 95% of the proteinaceous material in a sample. Such a protein may be prepared from the bacterial cells in which it naturally occurs, or may be prepared using standard recombinant DNA methodology to cause high levels of expression of the
10 protein in a bacterium or other cell in which it does not naturally occur, e.g., E. coli. A crude extract of such recombinant protein is included within the definition of purified protein.

Using standard techniques the enzyme described below
15 can be readily cloned, for example, by microsequencing of the protein or fragments thereof, preparation of oligonucleotides useful as probes for a library of clones generated from the nucleic acid of a desired organism, e.g., Rhodothermus marinus and screening of that library
20 with such probes to isolate fragments of DNA encoding the protein. Alternatively, an antibody to the protein may be produced and an expression library screened to determine which clone expresses an antigenic determinant recognized by that antibody. Other standard techniques are well
25 known to those of ordinary skill in the art to isolate such genes encoding the claimed proteins. Such genes encode recombinant alkaline phosphatase.

Thus, in a second aspect the invention features recombinant alkaline phosphatase having the above

properties, and cells encoding nucleic acid including such recombinant DNA. Equivalent genes encoding such phosphatases can be cloned using standard methodology.

In a third aspect, the invention features a method for
5 use of the above enzymes in labelling of protein or nucleic acid, and in various molecular biology techniques. Thus, the enzymes of the present invention may be used in standard labelling reactions and in diagnostic assays. They may be also used in molecular biology techniques in
10 which removal of a phosphate group is desired.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

15 Description of the Preferred Embodiment

The drawings will first briefly be described.

Drawings

Fig. 1 is a reproduction of a SDS-PAGE gel of
20 purified Rhodothermus marinus alkaline phosphatase. Lane 1 represents molecular weight markers. Lanes 2, 3, 4, and 5 represent 0.4, 0.8, 1.6 and 3.2 ug of purified protein, respectively.

Fig. 2 is a graphical representation of the
25 temperature activity profile of the enzyme. Temperature is presented on the x-axis. Specific activity (u/mg) is presented on the y-axis.

Fig. 3 is a graphical representation of the stability of the enzyme at 65°C. Incubation at 65°C (hours) is

presented on the x-axis. Percent initial activity is presented on the y-axis.

Fig. 4 is a graphical representation of the pH activity profile of the enzyme. Open squares represent enzyme activity assayed in 0.1M CAPS, 1mM MgCl₂ and 6mM pNPP. Solid diamonds represent enzyme activity assayed in 0.1M each of ethanolamine, diethanolamine, triethanolamine, 1mM MgCl₂ and 6mM pNPP. On the x-axis pH is presented. Specific activity (u/mg) is presented on the y-axis.

Fig. 5 is a representation of non-isotopic detection of various amounts (0.01 ng, 0.05 ng, 0.1 ng, 0.5 ng, 1 ng, 5 ng, 10 ng) of dot blotted DNA with Rhodothermus marinus alkaline phosphatase.

Fig. 6 represents a Southern Blot of human genomic DNA probed with an N-ras DNA fragment labelled with cloned *Rhodothermus marinus* alkaline phosphatase. Four amounts of total DNA were loaded: 1.0, 0.5, 0.2, 0.1µg. Target DNA is equivalent to 0.5, 0.25, 0.1, 0.05 pg.

Fig. 7 represents a sequence of genomic DNA from *Rhodothermus marinus*, containing the gene for alkaline phosphatase and flanking regions (SEQ. ID. NO. 1).

Fig. 8 represents a sequence of plasmid pCRM1.8, containing a 1.8kb segment of genomic DNA containing alkaline phosphatase and flanking sequences from *Rhodothermus marinus*, cloned into pUC19 (SEQ. ID. NO. 2).

Fig. 9 represents a presumptive full-length protein sequence (455 amino acids) of Rma alkaline phosphatase (SEQ. ID. NO. 4) and the corresponding nucleic acid

sequence (1368 bp) (SEQ. ID. NO. 3). The first 20 amino acids are presumed to function as a signal sequence in E. coli. The underlined portion of the amino acid sequence has been verified by protein sequencing of cloned enzyme
5 isolated from E. coli.

Example: Alkaline Phosphatase from Rhodothermus Marinus

The following is an example of Rhodothermus marinus
10 alkaline phosphatase isolation and characterization. This is not limiting in the invention.

Culture conditions:

Rhodothermus marinus, strain ATCC 43812 was grown
15 under aerobic conditions at 65°C as described by Alfredsson et. al. (1988) "Rhodothermus marinus, gen. nov., sp. nov., a Thermophilic, Halophilic Bacterium from Submarine Hot Springs in Iceland" J. Gen. Microbiology 134, 299-306. Cells were harvested by
20 continual flow centrifugation and stored at -80°C.

Activity assay:

Alkaline phosphatase activity was monitored spectrophotometrically at 405nm by following the
25 conversion of p-nitrophenylphosphate (pNPP) to p-nitrophenol. One unit is defined as the amount of enzyme required to release one micromole of p-nitrophenol in one minute. All assays were performed in 100mM CAPS (3-(cyclohexylamino)-1-propanesulfonic

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acid) pH10, 15% glycerol, 1mM MgCl₂, and 6mM pNPP at 37°C unless otherwise specified.

Purification:

5 Frozen cells (33g) were resuspended in 25mM Tris-HCl
pH7.4, 50mM NaCl, 1mM MgCl₂, and 0.1% Triton* X-100 (100ml
final volume) and lysed by sonication. The lysate was
cleared of cellular debris by centrifugation, supplemented
to a final NaCl concentration of 350mM and chromatographed
10 on DE 52 anionic exchange resin (Whatman; 4g resin per g
cells) equilibrated in 25mM Tris-HCl pH 7.4 and 350mM
NaCl. The majority of alkaline phosphatase activity
appeared in the flow through which was diluted in 25mM
Tris-HCl pH 7.4 to lower the salt concentration to 100mM.
15 The diluted flow through was applied to a column of
Heparin Sepharose* CL-6B resin (Pharmacia) equilibrated in
25mM Tris-HCl pH7.4 and 100mM NaCl. Alkaline phosphatase
activity did not bind to the resin. The flow through was
next applied to a Q-Sepharose* FF column (Pharmacia)
20 equilibrated in 25mM Tris-HCl pH 7.4 and 100mM NaCl. The
column was developed with a linear gradient from 100 to
350mM NaCl in 25mM Tris-HCl pH 7.4. Alkaline phosphatase
eluted at ~ 250mM NaCl. Appropriate fractions were pooled,
supplemented to 1mM MgCl₂, and resolved on hydroxylapatite
25 (BIO-RAD) equilibrated in 25mM Tris-HCl pH 7.4 and 10mM
NaCl (Buffer A). The enzyme activity was eluted with a
linear gradient from 100% Buffer A to 100% Buffer B (50mM
Na phosphate pH 7.0). Fractions containing alkaline
phosphatase activity were pooled, equilibrated into 50mM

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Tris-HCl pH 9.3 and 25mM NaCl (Buffer A) on Centriprep-30* concentrators (Amicon) and applied to a Q-Sepharose FF column equilibrated in Buffer A. The enzymatic activity was eluted with a linear gradient from 25 to 600mM NaCl in Buffer A. Pooled fractions were exchanged into 25mM Tris-HCl pH 7.4 and 25mM NaCl as described above and applied to a DEAE-Sephacel* column (Pharmacia) in the same buffer. Alkaline phosphatase was eluted with a linear gradient from 25 to 300mM NaCl in 25mM Tris-HCl. Appropriate DEAE-Sephacel fractions were combined, exchanged into 25mM Tris-HCl, 25mM NaCl and 50mM MgCl₂ on a Centriprep 30 apparatus and heated at 100°C for 10 minutes. Insoluble, heat-denatured proteins were removed by centrifugation.

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Characterization:

Following the above purification procedure analysis of the preparation by SDS-PAGE revealed that enzyme subunits corresponded to two closely spaced polypeptides which migrated at an apparent molecular weight of approximately 55,000 daltons (Fig. 1). The gel used was purchased from BioRad as a Mini-Protein II Ready Gel*, Catalogue No. 161-0902, 4-15% gradient gel and used according to the manufacturer's specifications. The presence of two protein bands, both of which display enzyme activity, may represent isozymes of the same protein, a post-translationally modified form of the enzyme, a partial proteolytic cleavage or possibly an artifact of the purification procedure. If the latter is true, then

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those skilled in the art recognize that the enzyme may be purified by other procedures which may or may not yield two polypeptides.

The final product represented a greater than 350-fold
5 purification of the crude cell lysate as determined by specific activity studies. The enzyme preparation was further characterized by determining the temperature and pH of optimal enzyme activity. To determine the temperature of optimal enzyme activity, stock alkaline
10 phosphatase (0.32 mg/ml) was diluted 250-fold in 25mM Tris-HCl pH7.4, 25mM NaCl and 1mM MgCl₂ and assayed for activity under standard conditions (see above) at the indicated temperatures. Results are presented in Fig. 2. To determine the pH of optimal enzyme activity, stock
15 alkaline phosphatase was diluted 200-fold in 25mM Tris-HCl pH 7.4, 25mM NaCl and 1mM MgCl. Enzyme assays were performed (as above) at the indicated pH values at 37°C in either 0.1M CAPS, 1mM MgCl₂ and 6mM pNPP or 0.1M each of ethanolamine, diethanolamine, triethanolamine, 1mM MgCl₂
20 and 6mM pNPP. Results are presented in Fig. 4. To determine enzyme stability at 65°C, stock alkaline phosphatase was diluted 20-fold in 25mM Tris-HCl pH 7.4, 25mM NaCl, 1mM MgCl₂ and 15% glycerol and incubated at 65°C for the indicated times. Samples were diluted an
25 additional 10-fold prior to activity assays performed under standard conditions. Results are presented in Fig. 3.

The alkaline phosphatase was also used in the non-isotopic detection of dot-blotted DNA (Fig. 5). Target

DNA (λ -Hind III) was denatured in TE + 0.2M NaOH and spotted in the various amounts (0.01 ng, 0.05 ng, 0.1 ng, 0.5 ng, 1 ng, 5 ng, 10 ng) onto a nylon membrane (Biodyne A; Pall) and fixed by baking for 1 hour at 80°C. The membrane was prehybridized for 1.5 to 2 hours at 65°C in ECL Gold hybridization buffer (Amersham) supplemented with 400mM NaCl, 4% casein and 50mM MgCl₂. Probe was prepared by crosslinking *R. marinus* alkaline phosphatase to λ -Hind III DNA. Equal volumes (typically 10ul each) of heat denatured probe (10ng/ul in 10mM Na phosphate pH 5.5), alkaline phosphatase (60ng/ml in 10mM Na phosphate pH 5.5) and glutaraldehyde (1.5%) were mixed and incubated at 37° for 45 minutes. The enzyme/probe complex was added directly to the prehybridization reaction (10ng probe/ml hybridization buffer) and allowed to hybridize overnight at 65°C. Following hybridization, the membrane was washed as follows: 2X SSC, 25mM Tris pH 7.4, 10mM MgCl₂, 0.1% SDS (3 x 5 minutes at room temperature); 0.2X SSC, 25mM Tris pH 7.4, 10mM MgCl₂ (3 x 5 minutes at 37°C); 0.1 M CAPS pH 10, 15% glycerol, 2mM MgCl₂ (30 minutes at room temperature). After washing the membrane was placed in a seal-a-meal bag with ~ 20ul CDP-Star substrate (Tropix) per cm² membrane and exposed to autoradiography film for 1 minute.

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Cloning of *Rhodothermus marinus* Alkaline Phosphatase

Genomic DNA was isolated from *Rhodothermus marinus* using standard procedures. Chromosomal DNA was subjected to partial *Sau* 3AI digestion and resolved by gel

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electrophoresis in low-melt agarose. Fragments ranging from 4-9 kb were isolated from the gel and ligated into pBluescript^{*} SK+ (Stratagene) which had been digested with *Bam*HI and dephosphorylated. Following ligation, DNA was
5 transformed into ultracompetent *E. coli* strain XL2-Blue MRF' (Stratagene) and plated on LB agar plates containing 100 ug/ml ampicillin, 50 ug/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate, a chromogenic indicator) and 5mM IPTG (isopropyl- β -D-thiogalactopyranoside). The plates were
10 incubated overnight at 37°C. A single blue colony (pBRM1) was observed out of approximately 6000 transformants.

Clone pBRM1

Restriction analysis of clone pBRM1 indicated an
15 insert of approximately 6 kb which encoded the alkaline phosphatase activity in cell lysates. No significant enzyme activity was observed in parallel studies performed with empty pBluescript vector. The alkaline phosphatase activity was found to be thermostable, as greater than 75%
20 of enzyme activity was retained after heating for 10 minutes at 100°C. In-gel colorimetric assays performed on cell lysates which had been renatured following SDS-PAGE indicated that the enzymatic activity was localized to a polypeptide which migrated at an apparent molecular weight
25 of ~46,000 Daltons. pBRM1 was transformed into *E. coli* CC118 [ara D139 Δ (ara, leu) 7697 Δ lac X74 phoA Δ 20 galE galK thi rpsE rpoB arg Eam recA1] and grown on BCIP indicator plates. This *E. coli* strain contains a deletion in the alkaline phosphatase (pho A) gene. The development

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of a blue colony indicated that the cloned enzyme is distinct from native *E. coli* alkaline phosphatase (BAP).

Clone pBRM1/KpnI

5 Mapping, deletion and expression studies indicated that approximately one half of the 6 kb insert in pBRM1 could be deleted without affecting enzyme activity. The smaller clone containing an insert of ~3 kb was designated as pBRM1/KpnI (SEQ ID NO. 1. See Figure 7). To
10 facilitate DNA sequencing, pBRM1/KpnI was digested with SacII to yield 0.45, 1.9 and 0.5 kb fragments which were individually subcloned into pBluescript.

Clone pCRM1.8

15 BLAST analysis (Gish et al., (1993) Nat. Genet. 3:266-72) of the DNA sequence data indicated that ~1 kb of the insert did not code for alkaline phosphatase and thus could be deleted. pBRM1/KpnI was digested with HindIII/KpnI to release a 1.8kb fragment which was
20 subsequently subcloned into pUC19. The resultant clone was designated as pCRM1.8 (SEQ ID NO. 2. See Figure 8).

Expression and Purification of Cloned Alkaline Phosphatase

An overnight culture of *E. coli* strain JM109 containing the
25 recombinant plasmid pCRM1.8 was diluted 1:40 in LB + 100 ug/ml ampicillin and grown with shaking at 37°C until the A_{600} reached 1.30. The culture was induced by the addition of 2.5 mM IPTG and allowed to incubate for an additional 18 hours. The cells were pelleted by centrifugation at

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6000 X g for 20 minutes at 4°C and stored at -80°C. Frozen cells (12g) were thawed and resuspended in 60 ml of lysis buffer (25 mM Tris, HCL pH 7.4, 50 mM NaCl, 1 mM MgCl₂, 1 mM EDTA, 0.05% Triton X-100 and 1 mg/ml lysozyme). The resuspension was stirred occasionally for 45 minutes at 4°C, sonicated to reduce viscosity and clarified by centrifugation at 70,000 X g for 30 minutes at 4°C. The supernatant was supplemented to a final concentration of 50 mM MgCl₂ and heated at 80°C for 20 minutes to precipitate heat labile proteins which were subsequently removed by centrifugation as above. The supernatant was diluted 1:1 with Buffer A (25 mM Tris, HCL pH 7.4, 20 mM NaCl and 1mM MgCl₂) to reduce salt concentration and chromatographed on DE 52 anionic exchange resin (Whatman). The column was developed with a 6-column volume linear gradient from 20 to 500mM NaCl in Buffer A. Alkaline phosphatase activity eluted at ~180 mM NaCl. Appropriate fractions were pooled and applied to a hydroxylapatite column (Bio-gel^{*} HTP; BioRad) equilibrated in 25 mM Tris•HCL pH 7.4 and 20 mM NaCl (Buffer B). The column was washed with two column volumes of Buffer B to remove residual MgCl₂ and then developed with an 8-column volume linear gradient of Buffer A to 150 mM Na phosphate pH 7.0. Alkaline phosphatase eluted at a phosphate concentration of ~40 mM. Pooled fractions were diluted 1:1 in Buffer B and applied to a Q Sepharose FF anion exchange column (Pharmacia). The column was washed with Buffer B to remove residual phosphate and then developed with a 10-column volume linear gradient of 20 to 500 mM NaCl in Buffer A.

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Enzyme activity was found to elute at ~250mM NaCl. The final product was found to be greater than 90% pure as determined by SDS-PAGE with a specific activity of ~3000 units/mg. The cloned protein has an apparent molecular weight which is less than that of native protein isolated from *R. marinus*. Protein sequencing of the amino terminus of both proteins revealed that the majority of the mature alkaline phosphatase isolated from *R. marinus* begins at serine residue 22 (SEQ ID NO. 4., See Figure 9), whereas the protein isolated from *E. coli* begins at glutamine residue 21. The difference in apparent molecular weight between the two proteins therefore is not due to a truncation at the amino terminus of the cloned protein. Amino acids upstream of these residues are presumed to function as signal peptides, as alkaline phosphatase is a periplasmic enzyme in *E. coli* and other bacteria.

DNA Probe Labelled with Cloned *Rhodothermus marinus* alkaline phosphatase

Cloned alkaline phosphatase was used for the non-isotopic detection of human genomic DNA (Fig. 6) The N-ras gene is easily detected in a 0.1 µg sample of total human genomic DNA by Southern blot analysis. The blot was prepared by fractionating a *Hind* III digestion of genomic DNA through a 1.0% agarose gel followed by capillary transfer onto a Hybond N membrane (Amersham) using 10 X SSC buffer. The membrane was prehybridized for 1.5 to 2 hours in modified ECL Gold hybridization buffer (from Amersham, 2M urea in place of 6M urea, 0.2% SDS in place

of 0.4% SDS.) supplemented with 400 mM NaCl, 4% casein and 20 mM MgCl₂. The probe was prepared by cross-linking cloned *R. marinus* alkaline phosphatase to N-ras DNA. Equal volumes (typically 10 μ l each) of heat denatured probe
5 (10ng/ml in water), cloned alkaline phosphatase diluted immediately prior to use (40 ng/ml in 10 mM MES buffer pH 5.0) and formaldehyde (2% in water) were mixed and incubated at 37°C for 30 minutes. The probe was added directly to the prehybridization reaction (5 ng/ml
10 hybridization buffer) and allowed to hybridize overnight at 50°C. Following hybridization, the membrane was washed as follows: one rinse and then 2 x 10 minute washes with the following buffer preheated to 50°C (washing was done on a shaker platform at RT): 100 mM Na phosphate pH 7.0, 150
15 mM NaCl, 2M urea, 0.4% SDS, 10mM MgCl₂. This was followed by one rinse and then 2 x 5 minute washes with the following buffer preheated to 55°C (wash at RT): 100mM Na phosphate pH 7.0, 150 mM NaCl, 10 mM MgCl₂. The last rinse was done at RT with 50 mM Tris pH 10, 100 mM NaCl, 2 mM Mg
20 Cl₂. The membrane was placed in a sealed plastic bag with 20 μ l CDP-star substrate (Tropix) per cm² membrane and exposed to autoradiography film for 2 hours.

Uses

Alkaline phosphatases of this invention have several
25 potential uses in the numerous non-isotopic methods for the detection of proteins and nucleic acids. For example, the high pH optimum of this enzyme makes it suitable with dioxetane substrates which undergo rapid conversion to the luminescent form at alkaline pH. When using streptavidin

conjugated alkaline phosphatase on positively charged membranes, as in nucleic acid hybridization, a pH greater than 9.5 is preferred to give decreased background. In addition, the high thermostability of this alkaline phosphatase makes it useful for direct crosslinking to nucleic acid probes. Hybridization and subsequent washes can be carried out under stringent conditions (i.e., elevated temperatures or in the presence of denaturants such as 6M urea) without loss of enzyme activity.

Alkaline phosphatases from different organisms may (or may not) behave similarly during purification. The high pH optimum for activity cannot be exploited for purification per se, but see below for screening. The high temperature optimum will be useful in purifying such enzymes after cloning into hosts that grow at a moderate temperature, such as E. coli. Extracts from E. coli could be heat treated to precipitate all proteins that denature at elevated temperatures.

If an enzyme is desired which is stable at 65°-75°C, it is possible to enhance the chances of discovery of such an enzyme by trying to isolate novel organisms that grow well at those temperatures. One could also select for organisms that are tolerant of high pH. In addition, knowing that an alkaline phosphatase is desired, one can then screen organisms, or libraries of recombinant clones, for alkaline phosphatase activity by use of the compound 5-bromo-4-chloro-3-indolyl phosphate (BCIP). A blue color is obtained when the phosphate group is removed from this compound, making it very convenient to screen for

activity. A pH activity profile would then be prepared to determine whether the phosphate removing activity was an alkaline phosphatase.

Other embodiments are within the following claims.

5

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: AMERSHAM LIFE SCIENCE, INC.
26111 Miles Road
Cleveland, OH 44128
- (ii) TITLE OF INVENTION: THERMOSTABLE ALKALINE PHOSPHATASE
OF RHODOTHERMUS MARINUS
- (iii) NUMBER OF SEQUENCES: 4
- (iv) CORRESPONDENCE ADDRESS:
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(D) PROVINCE: Ontario
(E) COUNTRY: Canada
(F) POSTAL CODE: K1P 5Y6
- (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb
storage
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: IBM P.C. DOS 5.0
(D) SOFTWARE: Word Perfect 5.1
- (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER: To Be Assigned
(B) FILING DATE: 24 October 1996
(C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER: USSN 60/005,965
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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2936 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GGGTACCGGA	GCGGCCGGGG	TTGATGAGCG	TGGCGAAGCG	CTCGACGATC	CGGCCGCCCT	60
GCACACGCAC	GGCTGCCAGC	TCGATGATCC	GATCGGCAGG	CCCGGAGCCC	GTCGTCTCGG	120
TATCGACGAC	GACGAACGAA	ACGGCGTCGA	GCTGCATCCC	CTCAGTGGGC	TTTCGCCGAC	180
GGCTTCCTCC	CGCCGGAGGC	CGTCTTCGGT	CAGGGCTGGC	AGGTCGAAAC	CCATGAGCTT	240
CGGCCAGTAA	GCTGACCGTA	GTTCAGTCAT	TCTCGAAGCT	GCCCACCAGC	CGATTTCGGCG	300
GCACGGATCC	GAAGGCGTAC	AGATTGACGT	CCACAGCGGT	GTGGCCATTG	GAGAGTCCAG	360
CCCACCACGG	ACCCGGCGAC	CGATCAGCTC	GGTAACCACC	TCGGCCCAGG	TGTCGGGTTC	420
GGCCGTGGCC	TGCGCGACGA	GCGCCTGCTC	GTCGGCGCGC	AGGCTGTCGA	GCCCGAGCCA	480
GGCCTGCAGG	AGCGAGTCGG	GTCGCTCCGA	ACGGCGCAGT	GCCGGGATAA	GCCGTTCGTA	540
GGAAGCCTGC	ACGCGGGCCA	GCACTTCCGG	GTGCCAGTCG	TAGACGCCGC	GGCCGTCCAC	600
GTTGCGCCCC	AGCGACAGAC	CGCCCGTCTC	GTGGTCGGCC	ACCGAGACGA	CGAGCGTCTG	660
TCCGTTCGCG	CGGGCAAAGT	CGAGCGCCAC	GGCCACGGCT	TCATCGTAAG	CCAGCACTTC	720
GCGGACGTGG	GCGGCGGCGT	CGTTGGCATG	TCCGGCGTGG	TCGATGCGGC	TGCCCTCCAC	780
CATCAGGAAG	AAGCCGTCCG	GATCGTCCGC	CAGCAGCTCG	AGTGCCGCGC	GGGTCATCTC	840
GGCCAGCGTA	GGGGACCTCT	TACCGGGTAC	GACCGGTCAA	TCTCGTAGGG	CAGATGGCTT	900
GGCCCGAACA	GTCCCAGCAC	CGGTCTCCTC	ACCCCCCGGC	GGAAGTCGGC	GGCCGTACGC	960
ACTACCTGGT	AGCCCATCGC	TTCGGCTTCG	CGCAGCAGAT	TGCGGCCGTC	CTGTCTCCGT	1020
CCGCCCTCCG	CGGTCGGCAG	GAAATAGCTC	CAGCCACCCC	CCAGCAGCAC	ATCGACGCGT	1080
TGCGCCAGCA	TCTGCGCGGC	GATTTTCGCTT	TCCATGCGCG	CTGCGGCACA	TGGGCGGCGA	1140
AGGCGGCCGG	CGTGGCGTGT	GAGATCCGGC	TGGTCGCCAC	CAGCCCGGTC	GCCATCCCAC	1200
GCGCTTTTGC	CGCTTCGAGC	AACGTGGCGA	GTGGGCGTCC	GGCCGTATCG	ACGGAATCGC	1260
GCCGTTGTAG	GTCTTGACGC	CGCAGGCATA	GGCCGTGCGC	CCGGCGAGCC	GGAGTCGGTC	1320
ACCCGGCTGG	AGGCCGAAGC	AGTACGCACG	GCACCGGTCT	GAATGGCATC	CAGCGTCAGT	1380
TCCTCACGTC	CCAGCACGGC	CCGGGCATAG	TCACGGGCCA	TCGTGATGCT	GGCCGGACCA	1440
CACCCGTCGG	CAATCATCAG	GATCAGGTTC	TTCGGACGCG	GCGACTGGGC	CCGAGCCCCT	1500
TCTCCGACCC	CCAACAGCAT	CACCAAAAAA	CAGCAATATC	CAGCGCATTG	TCGATTTCGCT	1560
CCCATCTTGA	TGAACACGGG	CTGTTCAAAG	ATACGACAGA	TCGGCTTTCA	TCCACAGCGC	1620
CCGGATTATC	TACGGAAAGA	GCACCATAAA	AAAGCCAACC	ACCCGAACAC	CTGTCCACCT	1680

TGAGGGCCAA	CCCGGCCGGG	TTGCGACCTC	AACGCAGCAG	GCCAAACAGG	CCGCACAATC	1740
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AGCATCACTT	TCACCTCGGC	AATCCGGCTA	TCAAGCTTCG	CCTCCACCTC	GGCAATCCGG	1860
CTGTGAGCT	TCGCCTCCAC	TTCCGGCGATC	CGGCCGTCGA	GCTTCGCTTC	AACCTTGGAG	1920
ATCCGGCCAT	TAAACTTTGC	CTCTACCTCG	GCAATTCCGC	GGTCAAGCTT	CACCTCTACG	1980
GCAGCGATGC	GTTGTTCCAG	ATGCGACACC	TCCTACGCTG	AATCCGACGG	TCCACGCTTC	2040
GCCACCTCCT	CCGTGATCGC	GTCTGCTCCA	GCACGCGCTT	CCACCTCGGT	GATGCGATTG	2100
TCCAGGCGCT	TTTCGGTCTC	GGCGACCCGC	CGCGCGAAAC	GCTCCTCTAC	AATCCCCAGC	2160
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CAGGATACCC	GGCGCGGTTC	TTTGTCGGAA	GATCCGCATT	CGGGCCGCTC	GCCACGCGTC	2640
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CGCGCCAGC	TCTCCGGCGC	AAAAGTCCGG	CCCGTCGCAA	CGAGCCAGGC	CTGATAGGGG	2880
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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A)	LENGTH:	4454 base pairs
(B)	TYPE:	nucleic acid
(C)	STRANDEDNESS:	single
(D)	TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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GGACGCGGCG	ACTGGGCCCC	AGCCCCCTCT	CCGACCCCCA	ACAGCATCAC	CAAAAACAGC	1920
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AGATCGGCTT	TCATCCACAG	CCCCGGATAC	TACGGAAAGA	GCACCATAAA	AAAGCCAACC	2040
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CGCAAACAGC	GCCACAATCA	CCCCGATCTG	TCCCGCCAG	AAAATAAACA	TCCAGCGCAC	2160
CAGACTTGCA	TACCGCTCGC	TTAGCATCAC	TTTCACCTCG	GCAATCCGGC	TATCAAGCTT	2220
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CAACATACGA	GCCGGAAGCA	TAAAGTGTA	AGCCTGGGGT	GCCTAATGAG	TGAGCTAACT	2340
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CTCAAAGGCG	GTAATACGGT	TATCCACAGA	ATCAGGGGAT	AACGCAGGAA	AGAACATGTG	2580
AGCAAAGGCG	CAGCAAAGG	CCAGGAACCG	TAAAAAGGCC	GCGTTGCTGG	CGTTTTTTCCA	2640
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TTCTACGGGG	TCTGACGCTC	AGTGGAACGA	AAACTCACGT	TAAGGGATTT	TGGTCATGAG	3300
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CTAAAGTATA	TATGAGTAAA	CTTGGTCTGA	CAGTTACCAA	TGCTTAATCA	GTGAGGCACC	3420
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ACGCTCACCG	GCTCCAGATT	TATCAGCAAT	AAACCAGCCA	GCCGGAAGGG	CCGAGCGCAG	3600
AAGTGGTCCT	GCAACTTTAT	CCGCCTCCAT	CCAGTCTATT	AATTGTTGCC	GGGAAGCTAG	3660
AGTAAGTAGT	TCGCCAGTTA	ATAGTTTGCG	CAACGTTGTT	GCCATTGCTA	CAGGCATCGT	3720
GGTGTACGCG	TCGTGTTTGG	GTATGGCTTC	ATTCAGCTCC	GGTTCCCAAC	GATCAAGGCG	3780
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CAACTGATCT	TCAGCATCTT	TTACTTTTAC	CAGCGTTTCT	GGGTGAGCAA	AAACAGGAAG	4200
GCAAAATGCC	GCAAAAAAGG	GAATAAGGGC	GACACGGAAA	TGTTGAATAC	TCATACTCTT	4260
CCTTTTTTCAA	TATTATTGAA	GCATTTATCA	GGGTATTGT	CTCATGAGCG	GATACATATT	4320
TGAATGTATT	TAGAAAAATA	AACAAATAGG	GGTTCCGCGC	ACATTTCCCC	GAAAAGTGCC	4380
ACCTGACGTC	TAAGAAACCA	TTATTATCAT	GACATTAACC	TATAAAAATA	GGCGTATCAC	4440

GAGGCCCTTT CGTC

4454

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1368 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```

ATGCGCTGGA TATTGCTGTT TTTGGTGATG CTGTTGGGGG TCGGAGAAGG GGCTCGGGCC      60
CAGTCGCCGC GTCCGAAGAA CCTGATCCTG ATGATTGCCG ACGGGTGTGG TCCGGCCAGC      120
ATCACGATGG CCCGTGACTA TGCCCGGGCC GTGCTGGGAC GTGAGGAAGT GACGCTGGAT      180
GCCATTCAAG CCGGTGCCGT GCGTACTGCT TCGGCCTCCA GCCGGGTGAC CGACTCGGCC      240
GCCGGGGCGA CGGCCTATGC CTGCGGCGTC AAGACCTACA ACGGCGCGAT TGCCGTTCGAT      300
ACGGCCGGAC GCCCACTCGC CACGTTGCTC GAAGCGGCAA AAGCGCGTGG GATGGCGACC      360
GGGCTGGTGG CGACCAGCCG GATCTCACAC GCCACGCCGG CCGCCTTCGC CGCCCATGTG      420
CCGCAGCGCG CCATGGAAAG CGAAATCGCC GCGCAGATGC TGGCGCAACG CGTCCATGTG      480
CTGCTGGGGG GTGGCTGGAG CTATTTCTTG CCGACCGCGG AGGGCGGACG GAGACAGGAC      540
GGCCGCAATC TGCTGCGCGA AGCCGAAGCG ATGGGCTACC AGGTAGTGCG TACGGCCGCC      600
GACTTCCGCC GGGGGGTGAG GAGACCGGTG CTGGGACTGT TCGGGCCAAG CCATCTGCCC      660
TACGAGATTG ACCGCGACCC GGAAGAGGTC CCCTCGCTGG CCGAGATGAC CCGCGCGGCA      720
CTCGAGCTGC TGGCGGACGA TCCGGACGGC TTCTTCCTGA TGGTGGAGGG CAGCCGCATC      780
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GCACTGCGCC GTTCGGAGCG ACCCGACTCG CTCCTGCAGG CCTGGCTCGG GCTCGACAGC      1080
CTGCGCGCCG ACGAGCAGGC GCTCGTCGCG CAGGCCACGG CCGAACCCGA CACCTGGGCC      1140
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ACCGCTGTGG ACGTCAATCT GTACGCCTTC GGACCCGGCG CCGAACGGCT GGTGGGCAGC      1260
TTCGAGAATG ACGAACTCGG CCGCTTACTG GCCGAACCTA TGGGTTTCGA CCTGCCAGCC      1320
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```

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 455 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met Arg Trp Ile Leu Leu Phe Leu Val Met Leu Leu Gly Val Gly Glu

1	5	10	15												
Gly	Ala	Arg	Ala	Gln	Ser	Pro	Arg	Pro	Lys	Asn	Leu	Ile	Leu	Met	Ile
	20		25										30		
Ala	Asp	Gly	Cys	Gly	Pro	Ala	Ser	Ile	Thr	Met	Ala	Arg	Asp	Tyr	Ala
	35					40						45			
Arg	Ala	Val	Leu	Gly	Arg	Glu	Glu	Leu	Thr	Leu	Asp	Ala	Ile	Gln	Thr
	50					55					60				
Gly	Ala	Val	Arg	Thr	Ala	Ser	Ala	Ser	Ser	Arg	Val	Thr	Asp	Ser	Ala
65					70					75					80
Ala	Gly	Ala	Thr	Ala	Tyr	Ala	Cys	Gly	Val	Lys	Thr	Tyr	Asn	Gly	Ala
			85					90						95	
Ile	Ala	Val	Asp	Thr	Ala	Gly	Arg	Pro	Leu	Ala	Thr	Leu	Leu	Glu	Ala
		100						105					110		
Ala	Lys	Ala	Arg	Gly	Met	Ala	Thr	Gly	Leu	Val	Ala	Thr	Ser	Arg	Ile
	115						120					125			
Ser	His	Ala	Thr	Pro	Ala	Ala	Phe	Ala	Ala	His	Val	Pro	Gln	Arg	Ala
	130					135					140				
Met	Glu	Ser	Glu	Ile	Ala	Ala	Gln	Met	Leu	Ala	Gln	Arg	Val	His	Val
145					150					155					160
Leu	Leu	Gly	Gly	Gly	Trp	Ser	Tyr	Phe	Leu	Pro	Thr	Ala	Glu	Gly	Gly
			165						170				175		
Arg	Arg	Gln	Asp	Gly	Arg	Asn	Leu	Leu	Arg	Glu	Ala	Glu	Ala	Met	Gly
		180						185					190		
Tyr	Gln	Val	Val	Arg	Thr	Ala	Ala	Asp	Phe	Arg	Arg	Gly	Val	Arg	Arg
	195					200						205			
Pro	Val	Leu	Gly	Leu	Phe	Gly	Pro	Ser	His	Leu	Pro	Tyr	Glu	Ile	Asp
	210					215					220				
Arg	Asp	Pro	Glu	Glu	Val	Pro	Ser	Leu	Ala	Glu	Met	Thr	Arg	Ala	Ala
225					230					235				240	
Leu	Glu	Leu	Leu	Ala	Asp	Asp	Pro	Asp	Gly	Phe	Phe	Leu	Met	Val	Glu
			245						250				255		
Gly	Ser	Arg	Ile	Asp	His	Ala	Gly	His	Ala	Asn	Asp	Ala	Ala	Ala	His
		260					265					270			
Val	Arg	Glu	Val	Leu	Ala	Tyr	Asp	Glu	Ala	Val	Ala	Val	Ala	Leu	Asp
	275						280					285			
Phe	Ala	Arg	Arg	Asp	Gly	Gln	Thr	Leu	Val	Val	Ser	Val	Ala	Asp	His
	290					295					300				
Glu	Thr	Gly	Gly	Leu	Ser	Leu	Gly	Arg	Asn	Val	Asn	Gly	Arg	Gly	Val
305					310					315				320	
Tyr	Asp	Trp	His	Pro	Glu	Val	Leu	Ala	Arg	Val	Gln	Ala	Ser	Tyr	Glu
			325						330					335	
Arg	Leu	Ile	Pro	Ala	Leu	Arg	Arg	Ser	Glu	Arg	Pro	Asp	Ser	Leu	Leu
		340						345					350		
Gln	Ala	Trp	Leu	Gly	Leu	Asp	Ser	Leu	Arg	Ala	Asp	Glu	Gln	Ala	Leu
	355					360						365			
Val	Ala	Gln	Ala	Thr	Ala	Glu	Pro	Asp	Thr	Trp	Ala	Glu	Val	Val	Thr
	370					375					380				
Glu	Leu	Ile	Gly	Arg	Arg	Ala	Val	Val	Gly	Trp	Thr	Ser	Asn	Gly	His
385					390				395					400	
Thr	Ala	Val	Asp	Val	Asn	Leu	Tyr	Ala	Phe	Gly	Pro	Gly	Ala	Glu	Arg
		405						410					415		
Leu	Val	Gly	Ser	Phe	Glu	Asn	Asp	Glu	Leu	Gly	Arg	Leu	Leu	Ala	Glu
		420						425					430		

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Leu	Met	Gly	Phe	Asp	Leu	Pro	Ala	Leu	Thr	Glu	Thr	Leu	Arg	Arg	Glu
		435					440					445			
Glu	Ala	Val	Gly	Glu	Ser	His									
	450					455									

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CLAIMS:

1. An enzymatically active thermostable alkaline phosphatase or enzymatically active portion thereof, wherein said phosphatase has at least 75% identity in its amino acid
5 sequence to the enzymatically active portion of the alkaline phosphatase of Rhodothermus marinus as set forth in SEQ ID NO:4.
2. The alkaline phosphatase of claim 1, wherein the amino acid sequence of said phosphatase includes less than 10
10 conservative amino acid changes compared to said phosphatase of Rhodothermus marinus.
3. The alkaline phosphatase of claim 1, wherein the amino acid sequence of said phosphatase includes less than
10 additional amino acids compared to said phosphatase of
15 Rhodothermus marinus.
4. A purified enzymatically active portion of the thermostable alkaline phosphatase present in Rhodothermus marinus having a subunit molecular weight of about 46,000-
55,000 as determined by SDS-PAGE and stable to heating at
20 65°C for 1 hour.
5. A purified recombinant nucleic acid encoding a thermostable alkaline phosphatase as defined in any one of claims 1-3.
6. A cell comprising the recombinant nucleic acid of
25 claim 5.
7. Recombinant alkaline phosphatase from Rhodothermus marinus having a subunit molecular weight of about 46,000-
55,000 as determined by SDS-PAGE gel and stable to heating at 65°C for 1 hour.

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8. Method for detecting nucleic acid or protein in a sample comprising the step of providing a nucleic acid, protein, or antibody labelled with a thermostable alkaline phosphatase from Rhodothermus marinus as set forth in SEQ ID NO:4 which is stable to heating at 65°C for 1 hour.

9. A purified recombinant nucleic acid as set forth in SEQ ID NO:3 encoding the thermostable alkaline phosphatase as defined in claim 4.

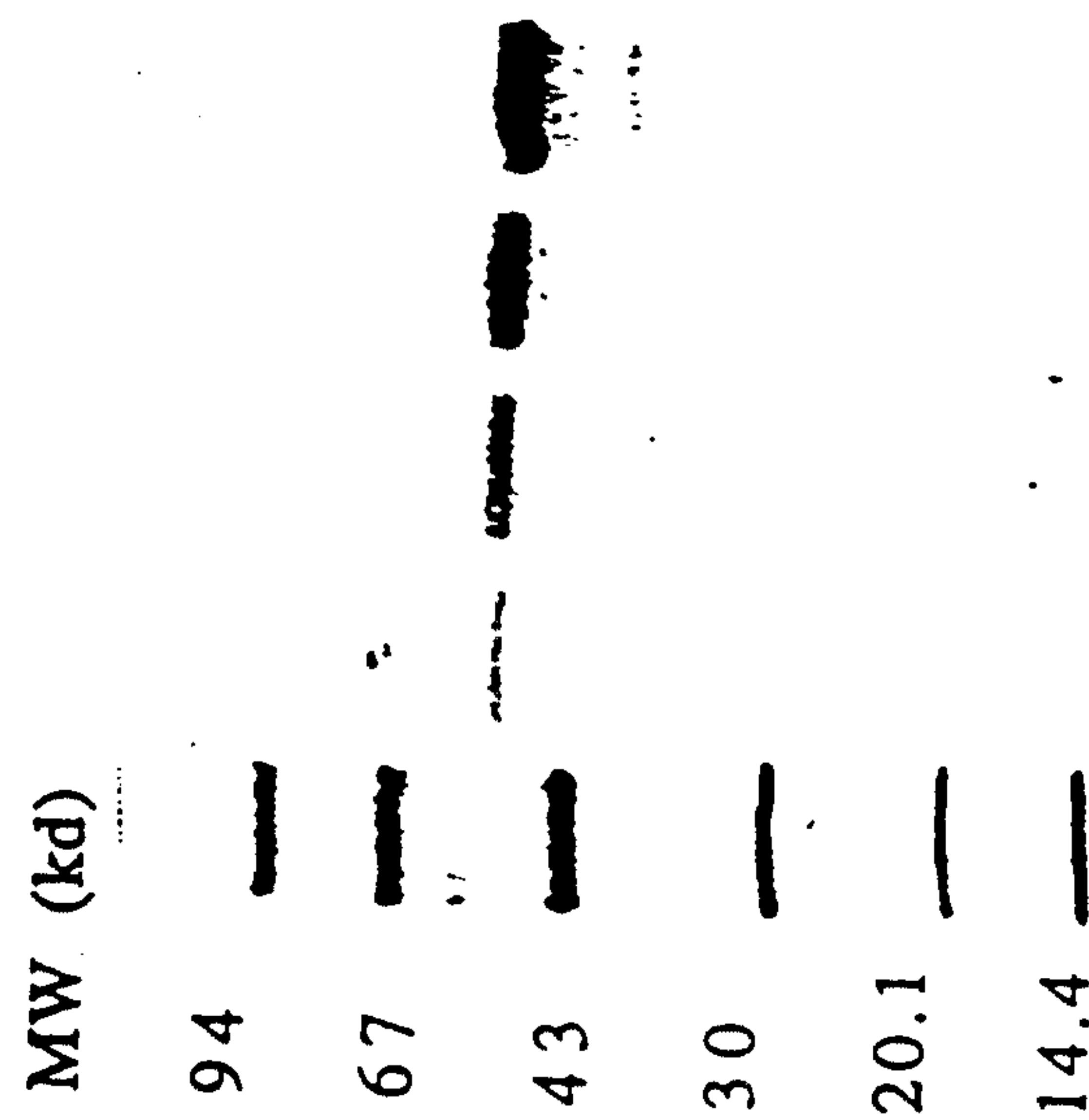
SMART & BIGGAR

OTTAWA, CANADA

PATENT AGENTS

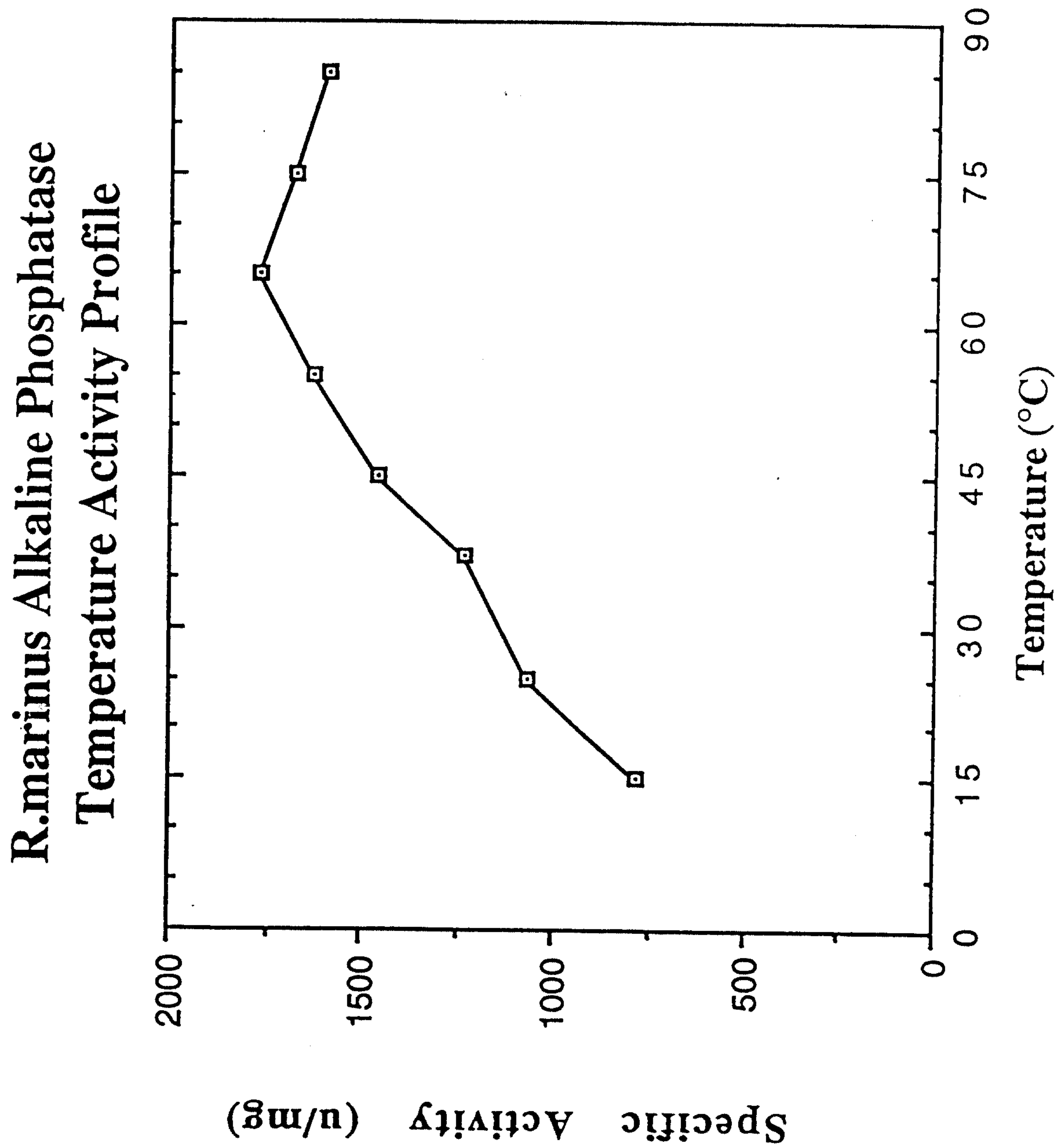
Fig. 1

R.marinus Alkaline Phosphatase
4-15% SDS-PAGE



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Fig. 2



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Fig. 3

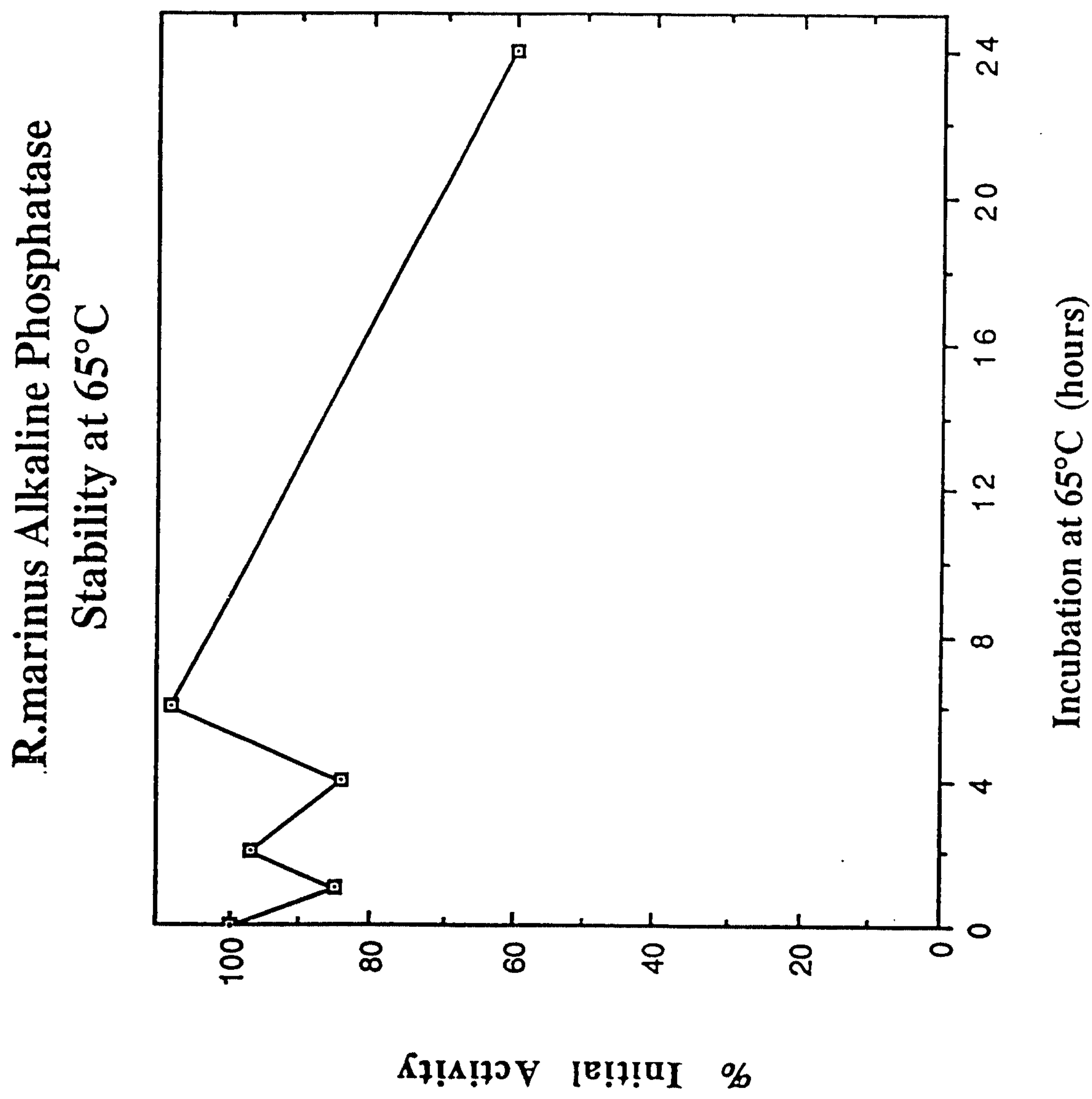


Fig. 4

R.marinus Alkaline Phosphatase
pH Activity Profile

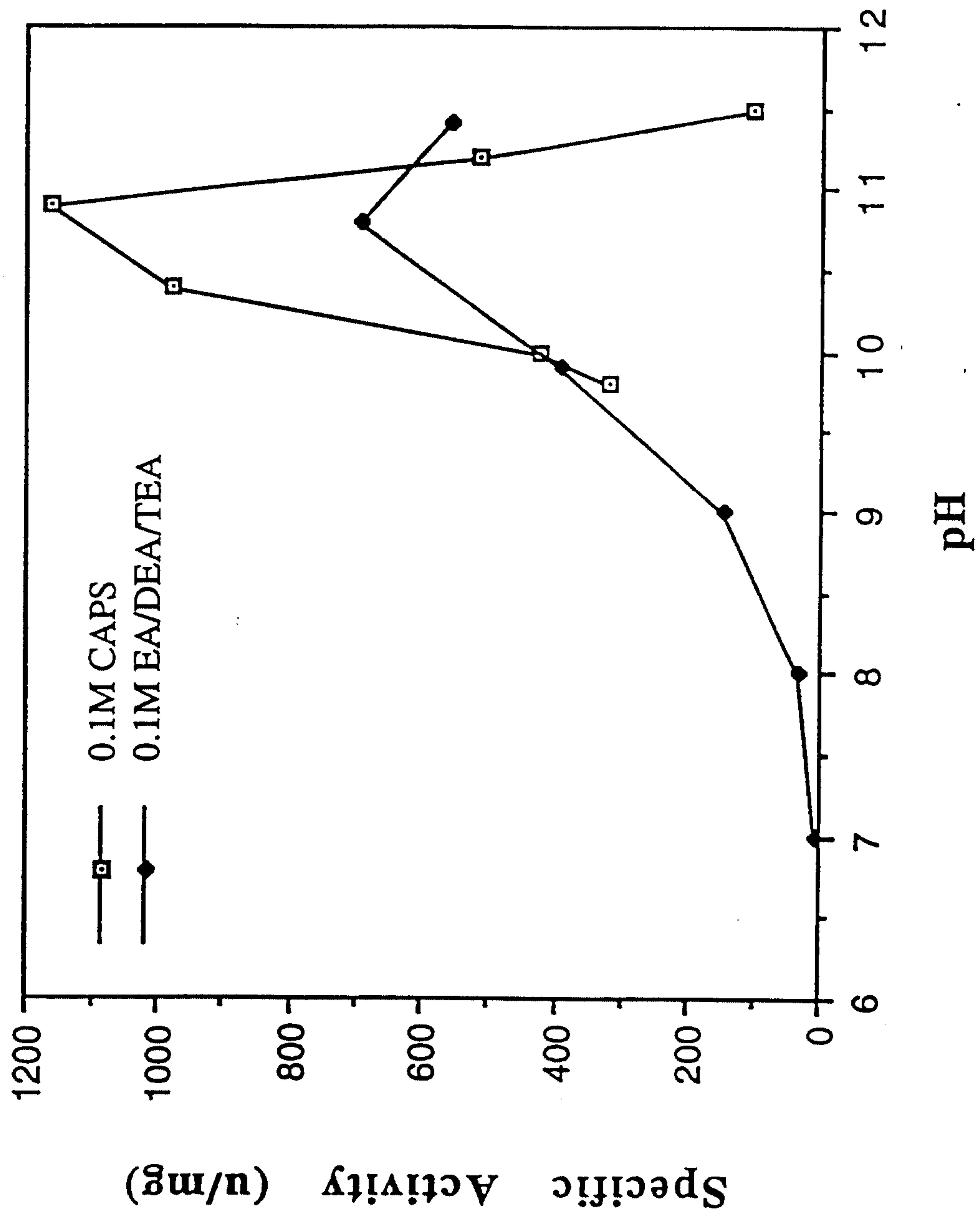


Fig. 5

**R. marinus Alkaline Phosphatase
Non-Isotopic Detection**

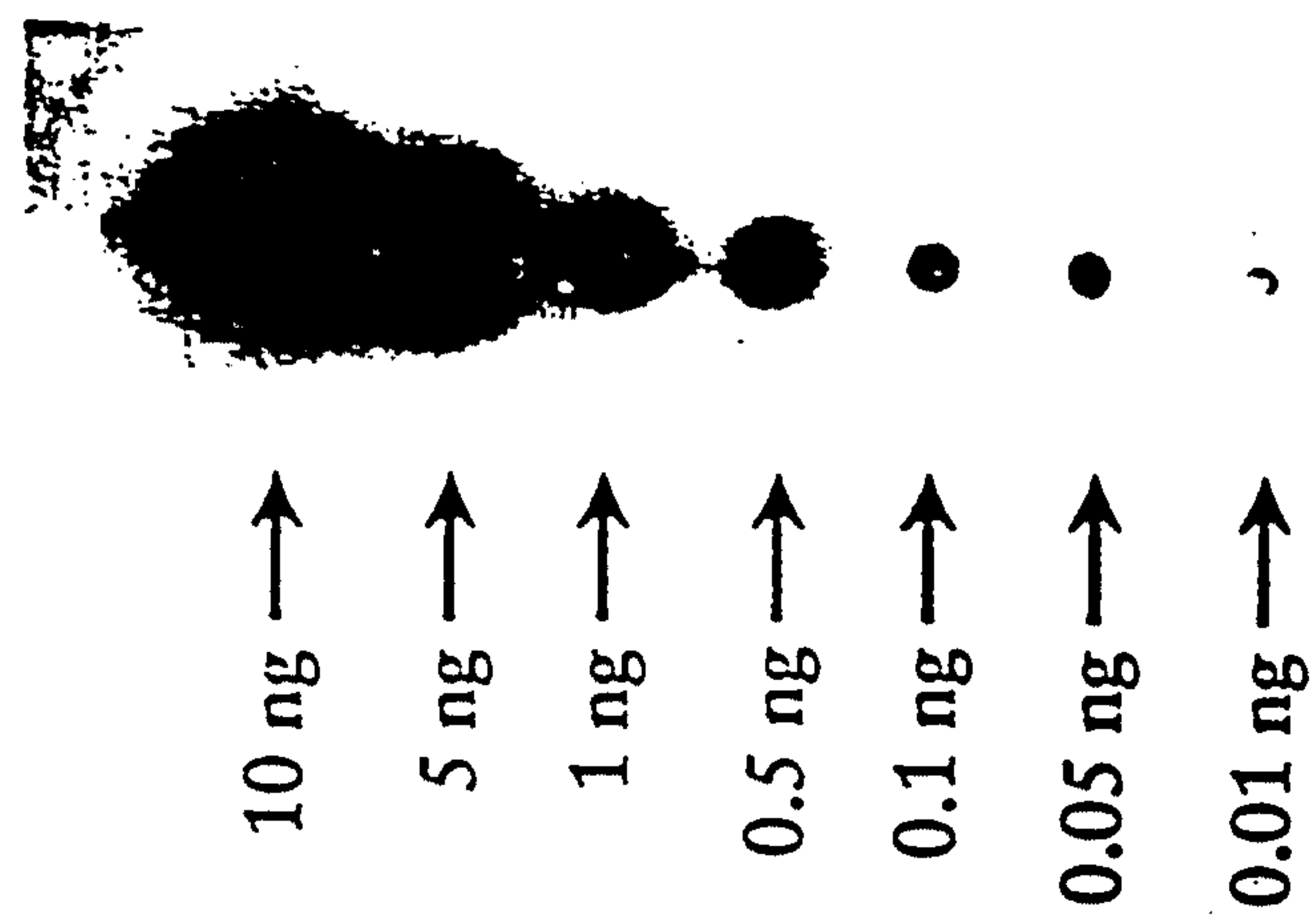
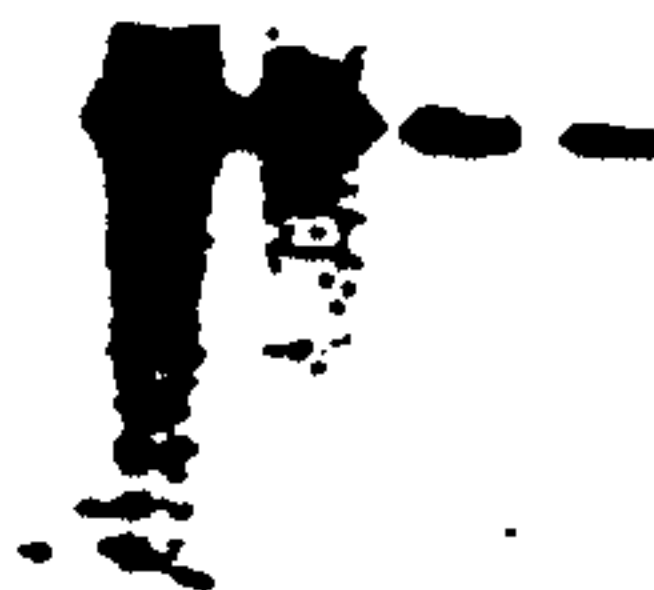


Fig. 6

Southern Blot

Human Genomic DNA/HindIII
N-ras 1.5 kb probe



1 0.5 0.2 0.1 ug

Sequence ID 1; 2936bp

Sequence of genomic DNA from *Rhodothermus marinus*, containing the gene for alkaline phosphatase and flanking regions.

GGGTACCGGAGCGGCCGGGGTTGATGAGCGTGGCGAAGCGCTCGACGATCCGGCCGCCCTGCACACGCACGGCTGCCAGC
TCGATGATCCGATCGGCAGGCCCGGAGCCCGTCTCGGTATCGACGACGACGAACGAAACGGCGTCGAGCTGCATCCC
CTCAGTGGGCTTTTCGCCGACGGCTTCCTCCCGCCGGAGGCCGTCTTCGGTCAGGGCTGGCAGGTCGAAACCCATGAGCTT
CGGCCAGTAAGCTGACCGTAGTTCAGTCATTCTCGAAGCTGCCCACCAGCCGATTTCGGCGGCACGGATCCGAAGGCGTAC
AGATTGACGTCCACAGCGGTGTGGCCATTTCGAGAGTCCAGCCCACCACGGACCCGGCGACCGATCAGCTCGGTAACCACC
TCGGCCCAGGTGTCGGGTTTCGGCCGTGGCCTGCGCGACGAGCGCCTGCTCGTTCGGCGCGCAGGCTGTCGAGCCCGAGCCA
GGCCTGCAGGAGCGAGTCGGGTTCGCTCCGAACGGCGCAGTGCCGGGATAAGCCGTTTCGTAGGAAGCCTGCACGCGGGCCA
GCACTTCCGGGTGCCAGTCGTAGACGCCGCGGCCGTCCACGTTGCGCCCCAGCGACAGACCGCCCGTCTCGTGGTTCGGCC
ACCGAGACGACGAGCGTCTGTCCGTTCGCGCGGGCAAAGTCGAGCGCCACGGCCACGGCTTCATCGTAAGCCAGCACTTC
GCGGACGTGGGCGGCGGCGTTCGTTGGCATGTCCGGCGTGGTCGATGCGGCTGCCCTCCACCATCAGGAAGAAGCCGTCCG
GATCGTCCGCCAGCAGCTCGAGTGCCGCGCGGGTTCATCTCGGCCAGCGTAGGGGACCTCTTACCGGGTACGACCGGTCAA
TCTCGTAGGGCAGATGGCTTGGCCCCGAACAGTCCCAGCACCGGTCTCCTCACCCCCCGGCGGAAGTCGGCGGGCCGTACGC
ACTACCTGGTAGCCCATCGCTTCGGCTTCGCGCAGCAGATTGCGGCGGTCTCCTGTCTCCGTCCGCCCTCCGCGGTTCGGCAG
GAAATAGCTCCAGCCACCCCCCAGCAGCACATCGACGCGTTGCGCCAGCATCTGCGCGGCGATTTTCGCTTTCCATGCGCG
CTGCGGCACATGGGCGGCGAAGGCGGCGCGGTGGCGTGTGAGATCCGGCTGGTCGCCACCAGCCCGGTTCGCCATCCCAC
GCGCTTTTTCGCGCTTCGAGCAACGTGGCGAGTGGGCGTCCGGCCGTATCGACGGAATCGCGCCGTGTAGGTCTTGACGC
CGCAGGCATAGGCCGTTCGCCCCGGCGAGCCGGAGTCGGTCACCCGGCTGGAGGCCGAAGCAGTACGCACGGCACCGGTCT
GAATGGCATCCAGCGTCAGTTCCTCACGTCCCAGCACGGCCCCGGGCATAGTCACGGGCCATCGTGATGCTGGCCGGACCA
CACCCGTTCGCAATCATCAGGATCAGGTTCTTCGGACGCGGCGACTGGGCCCCGAGCCCCCTTCTCCGACCCCCAACAGCAT
CACCAAAAAACAGCAATATCCAGCGCATTGTCGATTTCGCTCCCATCTTGATGAACACGGGCTGTTCAAAGATACGACAGA
TCGGCTTTTCATCCACAGCGCCCGGATTATCTACGGAAAGAGCACCATAAAAAAGCCAACCACCCGAACACCTGTCCACCT
TGAGGGCCAACCCGGCCGGGTTGCGACCTCAACGCAGCAGGCCAAACAGGCCGCAACAATCACCCCGATCTGTCCGCCAGA
AAATAAACATCCAGCGCACCCAGACTTGCATACCGCCGCTTAGCATCACTTTCACCTCGGCAATCCGGCTATCAAGCTTCG
CCTCCACCTCGGCAATCCGGCTGTTCGAGCTTCGCCTCCACTTCGGCGATCCGGCCGTTCGAGCTTCGCTTCAACCTTGGAG
ATCCGGCCATTAACTTTGCCTCTACCTCGGCAATTTCGGCGGTCAAGCTTCACCTCTACGGCAGCGATGCGTTGTTCCAG
ATGCGACACCTCCTACGCTGAATCCGACGGTCCACGCTTCGCCACCTCCTCCGTGATCGCGTCTGCTCCAGCACGCGCTT
CCACCTCGGTGATGCGATTGTCCAGGCGCTTTTCGGTCTCGGCGACCCGCCGCGCGAAACGCTCCTCTACAATCCCCAGC
AGATTGTTACGCTCGTGATGGGCCGCTCGTTGAGCAAATTGATGAGCGCCTCGACACCTTCGTCGCCGAGCTTTTTCACG
CAAAGCTTTTCACGCAAACTTTTCGGACGGTCAGATTGCCATGAGCGCCTCCACATAGGATAGGCTAAAAGGAAATCGCA
CCTTATTTCTGGAGGTTCCCGTTTTTCGTCCAAACCGTCCGGTGTCTCGTTGTCAATTGCCCGGCCAGGTTTCGGAGGCTAG
ATTCGAGCTGTCGGGCTAGCCAGGCGCCGTATTGCGGATTGGGCAGGACGATCCAGCGCGTGCCCCACCAGGAGCGATAA
CGCCGGACAGTGCTCGCCGTGCTTCGGCTCTGGTTTCAGGATCGACATAAAGTCGCCCAGCTGGTTCGCCGATCTGGAGC
AGGATGCGATAGCGCTGGCCCAGGATACCCGGCGCGGTTCTTTGTTCGGAAGATCCGCATTCGGGCGCTCGCCACGCGTC
AGGATTACGTTCGAGCGTGTACCGGCAGTGGGAAGCCGACGGCCTGCAGGTTACGACGGGTGGCCTCCTCCAGGTTCGGCCG
TGCGGTTGGTCACATAAAAGACCTGACGCCGTGCCGGCGCGCCTCCTGTACGAACACGACAGCACCGGGCACAGGTTTCGG
CCTGTGCAGCCTGCACCCAGCGCGCCAGCTCTCCGGCGCAAAGTCCGGCCCGTTCGCAACGAGCCAGGCCTGATAGGGG
CTGTTGTTCGAGCACGGTCTCGTCCACGTCCACGATCCACTAGTTCTAGAGCGGCCG

FIGURE 7

Sequence ID 2; 4454bp. Sequence of plasmid pCRM1.8, containing a 1.8kb segment of genomic DNA containing alkaline phosphatase and flanking sequences from *R. marinus*, cloned into pUC19.

TCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGAT
GCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTGGCGGGTGTGGGGCTGGCTTAACCTATGCCGGCATCAGA
GCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCC
ATTCGCCATTACAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGG
GGATGTGCTGCAAGGCGATTAAAGTTGGGTAAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAATT
CGAGCTCGGTACCGAGCGGCCGGGGTTGATGAGCGTGGCGAAGCGCTCGACGATCCGGCCGCCCTGCACACGCACGGCTG
CCAGCTCGATGATCCGATCGGCAGGCCCGGAGCCCGTCTCTCGGTATCGACGACGACGAACGAAACGGCGTCTGAGCTGC
ATCCCCTCAGTGGCTTTTCGCCGACGGCTTCTCCCGCCGGAGCGTCTCGGTTCAGGGCTGGCAGGTGCAAACCCATGAGTT
CGGCCAGTAAGCGGCCGAGTTCGTCAATTCTCGAAGCTGCCACCAGCCGTTTCGGCGCCGGGTCCGAAGGCGTACAGATTG
ACGTCCACAGCGGTGTGGCCATTTCGAGGTCCAGCCCACCACGGCCCCGGCGACCGATCAGCTCGGTAACCACCTCGGCCCA
GGTGTGGGGTTCGGCCGTGGCCTGCGCGACGAGCGCCTGCTCGTCCGGCGCGCAGGCTGTGAGCCCGAGCCAGGCCTGCA
GGAGCGAGTCGGGTCTGCTCCGAACGGCGCAGTGCCGGGATAAGCCGTTTCGTAGGAAGCCTGCACGCGGGCCAGCACTTCC
GGGTGCCAGTCGTAGACGCCGCGGCCGTTTACGTTGCGCCCCAGCGACAGACCGCCCGTCTCGTGGTTCGGCCACCGAGAC
GACGAGCGTCTGTCCGTGCGGGCGGGCAAAGTCGAGCGCCACGGCCACGGCTTCATCGTAAGCCAGCACTTCGCGGACGT
GGGCGGCGGCGTCTGTTGGCATGTCCGGCGTGGTTCGATGCGGCTGCCCTCCACCATCAGGAAGAAGCCGTCCGGATCGTCC
GCCAGCAGCTCGAGTGCCGCGCGGGTCATCTCGGCCAGCGAGGGGACCTCTTCCGGGTTCGCGGTCAATCTCGTAGGGCAG
ATGGCTTGGCCCCGAACAGTCCCAGCACCGGTCTCCTCACCCCCCGGCGGAAGTCGGCGGGCGTACGCACTACCTGGTAGC
CCATCGCTTCGGCTTCGCGCAGCAGATTGCGGCCGTCTGTCTCCGTCCGCCCTCCGCGGTTCGGCAGGAAATAGCTCCAG
CCACCCCCCAGCAGCACATGGACGCGTTGCGCCAGCATCTGCGCGGGCGATTTTCGCTTTCCATGGCGCGCTGCGGCACATG
GGCGGGCAAGGCGGCCGGCGTGGCGTGTGAGATCCGGCTGGTCCGACCAGCCCGGTTCGCCATCCCACGCGCTTTTGCCG
CTTCGAGCAACGTGGCGAGTGGGCGTCCGGCCGTATCGACGGCAATCGCGCCGTTGTAGGTCTTGACGCCGCGAGGCATAG
GCCGTGCCCCCGGCGGCCGAGTCGGTCACCCGGCTGGAGGCCGAAGCAGTACGCACGGCACCGGTCTGAATGGCATCCAG
CGTCAGTTCCTCACGTCCCAGCACGGCCCCGGGCATAGTCACGGGCCATCGTGATGCTGGCCGGACCACACCCGTTCGGCAA
TCATCAGGATCAGGTTCTTCGGACGCGGCGACTGGGCCCCGAGCCCCTTCTCCGACCCCCAACAGCATCACCAAAAACAGC
AATATCCAGCGCATTTGTGCATCGCTCCATCTGATGAACACGGGCTGTTCAAAGATACGACAGATCGGCTTTTCATCCACAG
CCCCGGATACTACGGAAAGAGCACCATAAAAAAGCCAACCACCCGAACACCTGTCCACCTTGAGGGCCAACCCGGCCGGG
TTGCGACCTCAACGCAGCAGCGCAAACAGCGCCACAATCACCCCGATCTGTCCCGCCCAGAAAATAAACATCCAGCGCAC
CAGACTTGCATACCGCTCGCTTAGCATCACTTTACCTCGGCAATCCGGCTATCAAGCTTGGCGTAATCATGGTTCATAGC
TGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAGCCTGGGGT
GCCTAATGAGTGAGCTAACTACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCTGTGCCAGCT
GCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTTCGTATTGGGCGCTCTTCGGCTTCTCTGCTCACTGACTCGC
TGCGCTCGGTCTGTTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGAT
AACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCA
TAGGCTCCGCCCCCTGACGAGCATCAGAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGAT
ACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCTGCGGCTTACCGGATACCTGTCCGCCTTT
CTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCTGTTCCGCTCCAAGCT
GGGCTGTGTGCAGAACCCCCCGTTTCAGCCCGACCGCTGCGCCTTATCCGGTAACCTATCGTCTTGAGTCCAACCCGGTAA
GACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTC
TTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGG
AAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTTGTTTGCAAGCAGCAGATTA
CGCGCAGAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACCTCACGT
TAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAATAAATGAAGTTTTAAATCAAT
CTAAAGTATATATGAGTAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTAT
TTCGTTTCATCCATAGTTGCCTGACTCCCCGTCTGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCT
GCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAG
AAGTGGTCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTA
ATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTACGCTCGTCTTGGTATGGCTTCATTCAGCTCC

FIGURE 8-A

GGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGT
TGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCG
TAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGC
CCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCG
AAAACCTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTT
TTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAGGGAATAAGGGCGACACGGAAA
TGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATT
TGAATGTATTTAGAAAAATAACAAATAGGGGTTCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCA
TTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTC

FIGURE 8-B

2188727

Sequence ID 3; 1368bp DNA sequence, Sequence ID 4; 455 amino acid sequence.
Presumptive full length sequence of Rma alkaline phosphatase. The first 20 amino acids are presumed to function as a signal sequence in *E. coli*. The underlined portion of the amino acid sequence has been verified by protein sequencing of cloned enzyme isolated from *E. coli*.

1/1	31/11
ATG CGC TGG ATA TTG CTG TTT TTG GTG ATG	CTG TTG GGG GTC GGA GAA GGG GCT CGG GCC
M R W I L L F L V M	L L G V G E G A R A
61/21	91/31
CAG TCG CCG CGT CCG AAG AAC CTG ATC CTG	ATG ATT GCC GAC GGG TGT GGT CCG GCC AGC
<u>Q S P R P K N L I L</u>	<u>M I A D G C G P A S</u>
121/41	151/51
ATC ACG ATG GCC CGT GAC TAT GCC CGG GCC	GTG CTG GGA CGT GAG GAA CTG ACG CTG GAT
I T M A R D Y A R A	V L G R E E L T L D
181/61	211/71
GCC ATT CAG ACC GGT GCC GTG CGT ACT GCT	TCG GCC TCC AGC CGG GTG ACC GAC TCG GCC
A I Q T G A V R T A	S A S S R V T D S A
241/81	271/91
GCC GGG GCG ACG GCC TAT GCC TGC GGC GTC	AAG ACC TAC AAC GGC GCG ATT GCC GTC GAT
A G A T A Y A C G V	K T Y N G A I A V D
301/101	331/111
ACG GCC GGA CGC CCA CTC GCC ACG TTG CTC	GAA GCG GCA AAA GCG CGT GGG ATG GCG ACC
T A G R P L A T L L	E A A K A R G M A T
361/121	391/131
GGG CTG GTG GCG ACC AGC CGG ATC TCA CAC	GCC ACG CCG GCC GCC TTC GCC GCC CAT GTG
G L V A T S R I S H	A T P A A F A A H V
421/141	451/151
CCG CAG CGC GCC ATG GAA AGC GAA ATC GCC	GCG CAG ATG CTG GCG CAA CGC GTC CAT GTG
P Q R A M E S E I A	A Q M L A Q R V H V
481/161	511/171
CTG CTG GGG GGT GGC TGG AGC TAT TTC CTG	CCG ACC GCG GAG GGC GGA CGG AGA CAG GAC
L L G G W S Y F L	P T A E G G R R Q D
541/181	571/191
GGC CGC AAT CTG CTG CGC GAA GCC GAA GCG	ATG GGC TAC CAG GTA GTG CGT ACG GCC GCC
G R N L L R E A E A	M G Y Q V V R T A A
601/201	631/211
GAC TTC CGC CGG GGG GTG AGG AGA CCG GTG	CTG GGA CTG TTC GGG CCA AGC CAT CTG CCC
D F R R G V R R P V	L G L F G P S H L P
661/221	691/231
TAC GAG ATT GAC CGC GAC CCG GAA GAG GTC	CCC TCG CTG GCC GAG ATG ACC CGC GCG GCA
Y E I D R D P E E V	P S L A E M T R A A
721/241	751/251
CTC GAG CTG CTG GCG GAC GAT CCG GAC GGC	TTC TTC CTG ATG GTG GAG GGC AGC CGC ATC
L E L L A D D P D G	F F L M V E G S R I
781/261	811/271
GAC CAC GCC GGA CAT GCC AAC GAC GCC GCC	GCC CAC GTC CGC GAA GTG CTG GCT TAC GAT
D H A G H A N D A A	A H V R E V L A Y D
841/281	871/291
GAA GCC GTG GCC GTG GCG CTC GAC TTT GCC	CGC CGC GAC GGA CAG ACG CTC GTC GTC TCG
E A V A V A L D F A	R R D G Q T L V V S
901/301	931/311
GTG GCC GAC CAC GAG ACG GGC GGT CTG TCG	CTG GGG CGC AAC GTG AAC GGC CGC GGC GTC
V A D H E T G G L S	L G R N V N G R G V
961/321	991/331

FIGURE 9-A

2188727

TAC GAC TGG CAC CCG GAA GTG CTG GCC CGC GTG CAG GCT TCC TAC GAA CGG CTT ATC CCG	
Y D W H P E V L A R V Q A S Y E R L I P	
1021/341	1051/351
GCA CTG CGC CGT TCG GAG CGA CCC GAC TCG CTC CTG CAG GCC TGG CTC GGG CTC GAC AGC	
A L R R S E R P D S L L Q A W L G L D S	
1081/361	1111/371
CTG CGC GCC GAC GAG CAG GCG CTC GTC GCG CAG GCC ACG GCC GAA CCC GAC ACC TGG GCC	
L R A D E Q A L V A Q A T A E P D T W A	
1141/381	1171/391
GAG GTG GTT ACC GAG CTG ATC GGT CGC CGG GCC GTG GTG GGC TGG ACC TCG AAT GGC CAC	
E V V T E L I G R R A V V G W T S N G H	
1201/401	1231/411
ACC GCT GTG GAC GTC AAT CTG TAC GCC TTC GGA CCC GGC GCC GAA CGG CTG GTG GGC AGC	
T A V D V N L Y A F G P G A E R L V G S	
1261/421	1291/431
TTC GAG AAT GAC GAA CTC GGC CGC TTA CTG GCC GAA CTC ATG GGT TTC GAC CTG CCA GCC	
F E N D E L G R L L A E L M G F D L P A	
1321/441	1351/451
CTG ACC GAG ACG CTC CGG CGG GAG GAA GCC GTC GGC GAA AGC CAC TGA	
L T E T L R R E E A V G E S H *	

FIGURE 9-B